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ELISA-based %%%oligonucleotide%%% %%%ligation%%% assay

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Single-well genotyping of diallelic sequence variations by a two-color ELISA-based oligonucleotide ligation assay

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ABSTRACT

Single nucleotide substitutions and unique insertions/deletions are the most common form of DNA sequence variation and disease-causing mutation in the human genome. Because of the biological and medical importance of these variations, a wide array of methods have been developed for their typing. We have applied an approach that combines the amplification of polymorphic regions by the polymerase chain reaction (PCR) with a system for typing diallelic variants using an oligonucleotide ligation assay (OLA). In this report, we describe a significant advance in this technology that permits the typing of two alleles in a single microtiter well. By marking each of the allele-specific primers with a unique hapten, i.e. digoxigenin and fluorescein, each OLA reaction can be detected by using hapten specific antibodies that are labeled with different enzyme reporters, alkaline phosphatase or horseradish peroxidase. This system permits the detection of the two alleles using a high throughput format that leads to the production of two different colors. We demonstrate the specificity, sensitivity and ease of data interpretation with this system. Furthermore, we show that multiplex PCR/OLA not only increases the throughput of DNA typing but also increases its accuracy in typing diallelic sequence variations using an approach that can be broadly applied for human genome analysis (in evaluating genotype/phenotype links), in typing infectious agents and in forensic analysis.

INTRODUCTION

The typing of single nucleotide substitutions and unique insertions/deletions plays a major role in identifying mutated oncogenes, genetic and infectious diseases, in matching tissues prior to transplantation, and in forensic and paternity testing (1-5). After identification, many technologies are available for the rapid typing of known sequence variants. These include: (i) the hybridization of allele-specific oligonucleotides (ASO) during (6) or after (7) the amplification of DNA by the polymerase chain reaction (PCR), (ii) allele-specific priming of PCR assays (8), and

(iii) the amplification of DNA targets by PCR followed by mini-sequencing assays (9), genetic bit analysis (10), or oligonucleotide ligation assays (OLA, 11). Among these, ligation assays have several features that make them ideally suited for genotyping on a large-scale, e.g. their specificity, speed and automation as well as their compatibility with PCR (11-13).

Ligation assays are simple reactions that determine whether or not two adjacent primers become covalently joined by a DNA ligase when hybridized to a complementary target (genomic DNA or an amplified PCR product) (11-13). The joining of two oligonucleotide primers (~20mers) by DNA ligase is dependent on three events: (i) the hybridization of the primers to complementary sequences within the target, (ii) the juxtaposition of the primers on the target, i.e. they must lie directly next to one another in a 5' to 3' orientation with no intervening nucleotides, and (iii) the primers must have perfect base-pair complementarity with the target at the site of their join. At this site even a single nucleotide mismatch between the primers and target will inhibit primer joining. Since a number of factors control the specificity of these reactions, the conditions associated with the ligation can be relaxed to the point where any nucleotide variation, i.e. single nucleotide substitutions or unique insertion/deletion variations, can be typed using only a single set of assay conditions (11-15). This greatly simplifies the development of new assays since it eliminates the need to optimize the genotyping conditions with each new probe set. It also simplifies the development of multiplex assays, i.e. assays where more than one allele and/or locus are typed in a single tube or microtiter well (13-18). In this report, we describe a system for multiplex typing of diallelic nucleotide substitutions using enzyme-linked immunosorbent assays (ELISA) that increases the throughput as well as the accuracy of genotyping by ligation assays.

MATERIAL AND METHODS

Primer reagents

Oligonucleotide primers for PCR amplification and ligation typing were assembled using standard phosphoramidite chemistry on an Applied Biosystems 394 DNA synthesizer (Foster City, CA). Allele-specific ligation primers were modified with a 5' amino-hexylphosphate linker (Aminolink2, Applied Biosystems), and following deprotection digoxigenin or fluorescein was added to

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the 5'-end using *N*-hydroxysuccinimide (NHS) esters for these reporters (Boehringer Mannheim, Indianapolis, IN) as previously described (11). After modification, the allele-specific primers were purified by reverse-phase high-performance liquid chromatography. Joining primers for the ligation assay were synthesized on a 5'-Biotin-ON CPG Column (Clontech, Palo Alto, CA) and chemically phosphorylated using 5' Phosphate-ON (Clontech) according to the manufacturer's instructions. Amplification primers for polymorphic regions in exon 2 of the human steroid 5-alpha-reductase (SRD5A1) gene (GDB id: G00-193-189, CCCAAATCATTTAAGATAGGATTAC, ATGATGTGAACAA-GGCGGAGTTCAC), intron 8 of the human lipoprotein lipase (LPL) gene (GDB id: G00-191-079, TACACTAGCAATGTCT-AGGTGA, TCAGCTTTAGCCCAGAATGC) and a sequence-tagged site (STS) from human chromosome 16, STS160 (gb: U48593, TCCTATGACTCTATATTATG, GATACACACAGTTT-TCTCC) were used in testing this approach. To type known single nucleotide substitutions in these loci, sets of ligation primers were synthesized as follows: (i) SRD5A1: digoxigenin-ACATAATCG-CCATTGTACAT, fluorescein-ACATAATCGCCATTGTACAC, phosphorylated-GCCAACAGTGCCATAGGCTT-biotin; (ii) LPL: digoxigenin-CTGTCAGGACTGTTTAAATAC, fluorescein-CTG-TCAGGACTGTTTAAATAA, phosphorylated-TACATGATCAT-GCTGGGTAAT-biotin; (iii) STS 160: digoxigenin-GTC(A/G)TTAAACTTTGAATCTAT, fluorescein-GTC(A/G)TTAAACTT-TGAATCTAC, phosphorylated-GGACATGTCTTTTCTTCT-biotin.

PCR amplification

DNA samples from 40 families available through the Centre d'Etude du Polymorphisme Humaine (CEPH) were used for PCR amplification of the three target loci. All amplification reactions were performed in a 96-well microtiter plate thermal cycler (PTC 100, MJ Research, Watertown, MA). Genomic DNA (20 ng) was mixed with a standard PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂ and 0.001% gelatin) containing 40 µM of each of the four deoxynucleotide triphosphates, 0.5 µM of each primer and 10 U/ml *Taq* polymerase (Perkin-Elmer-Cetus, Norwalk, CT) and each reaction was covered with 50 µl mineral oil. This was followed by thermal cycling with an initial denaturation at 93°C for 1 min followed by 35 cycles of denaturation at 93°C for 30 s, primer annealing at 60°C for SRD5A1 and LPL, or 55°C for STS160 for 30 s, and primer extension at 72°C for 2 min. After 35 cycles, a final extension was carried out at 72°C for 5 min.

Oligonucleotide ligation assay

Following the amplification of target loci (SRD5A1, LPL or STS160), PCR products (~20 µl) were diluted with 50 µl distilled H₂O containing 0.1% Triton X-100. An aliquot of the diluted products (10 µl) was mixed with 10 µl of a solution containing 2x ligase buffer (40 mM Tris-HCl (pH 8.0)/20 mM MgCl₂/2 mM dithiothreitol), 2 mM nicotinamide adenine dinucleotide, 25 mM KCl, 0.167 U Ampligase DNA Ligase (Epicentre, Madison, WI), and 200 fmol of each of the ligation primers (the two allele-specific primers and the joining primer for the locus being tested). Ligation reactions were overlaid with mineral oil and placed in a thermocycler for 10 cycles at 93°C for 30 s and 58°C for 4 min. After cycling, the reactions were stopped by the addition of 10 µl 0.1 M EDTA in 0.1% Triton H₂O and transferred in their entirety

(including the mineral oil) to a 96-well flat bottom microtiter plate (Falcon) that had been coated with streptavidin (Sigma) (50 µl 25 µg/ml incubated 1 h at 37°C). Streptavidin plates were blocked with 200 µl/well of 0.5% bovine serum albumin (Sigma) in 1x PBS (ICN) for 30 min prior to use to eliminate non-specific binding sites within the wells. Ligation products were allowed to capture on the streptavidin plate at room temperature (RT) for 1 h, and the plate was washed twice with an NaOH buffer (0.01 M NaOH/0.05% Tween 20) followed by two washes with Tris buffer (100 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.05% Tween 20). An antibody mixture (40 µl in 1x PBS with 0.5% BSA) consisting of a 1 to 1000 dilution of alkaline phosphatase-labeled anti-fluorescein antibodies and 1 to 1000 dilution of horseradish peroxidase-labeled anti-digoxigenin antibodies was added to each well. After 30 min at RT, plates were washed six times with Tris buffer. After washing, an alkaline phosphatase substrate (25 µl/well, BRL ELISA amplification system) was added to the wells, the plates were incubated for an additional 10 min at RT, and then 25 µl of amplifier were added to each well. Spectrophotometric absorbances were taken at 490 nm using a microplate reader (BioRad 3550) and saved in the attached computer. After detection of the fluorescein reporter, the plates were washed again six times with Tris buffer and 50 µl of the horseradish peroxidase substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma), were added to each well to detect the digoxigenin reporter. Spectrophotometric absorbances were taken at 655 nm for this reporter and saved in the attached computer.

RESULTS AND DISCUSSION

To genotype a diallelic nucleotide substitution or unique insertion/deletion by a ligation assay, three oligonucleotide primers (~20 nucleotides in length) are employed, i.e. two allele-specific primers which differ only in sequence by the nucleotide or the inserted/deleted base(s) to be typed, and one joining primer that is located directly adjacent (3') to the variant position(s). A number of formats can be used to detect whether two adjacent primers have been covalently joined by DNA ligase following target hybridization. Sizing by gel electrophoresis is one approach because ligated primers are usually twice the size (~40 bases) of the unjoined primers (~20 bases). In this approach, substantial multiplexing can be obtained by labeling different ligation reactions with different reporters and/or by shifting their mobilities in the gel by adding standard or specialized nucleotides (13-17). However, the ultimate throughput of this approach can be limiting due to its dependence on gel electrophoresis for analysis. Furthermore, if the outcome of these reactions is suboptimal, data interpretation can be difficult since the presence of a fluorophore and the size of the product must be assessed.

Ligation assays can also be performed in formats that are not dependent on gel electrophoresis (11,12,18). One of these approaches uses time-resolved fluorophores to mark alternative alleles and a biotinylated joining primer to capture primers out of solution prior to their detection. Although this format has the advantage of typing two alleles in a single well, a specialized manifold is required for the readout (18). An alternative to this format is based on the detection of ligated products by an enzyme-linked immunosorbent assay (ELISA) (12). We have recently explored the use of different haptens and their specific antibodies to multiplex the detection of two ligation reactions in a single microtiter well. An overview of this concept is shown in

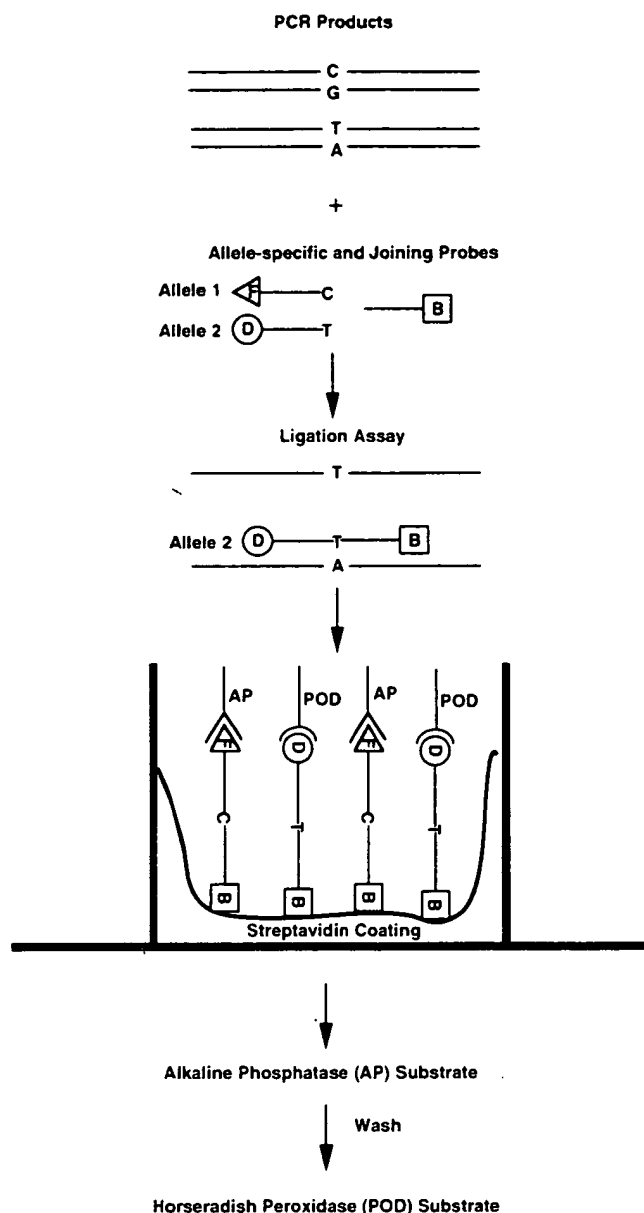


Figure 1. Multiplex ELISA-based OLA. Following the amplification of a region of the genome containing a diallelic sequence variation, PCR products are mixed with a joining probe (labeled with biotin) and two allele-specific probes [each labeled with a different hapten, fluorescein (F) or digoxigenin (D)]. Following ligation, the biotinylated joining probes are captured on streptavidin-coated microtiter wells and an ELISA is performed with alkaline phosphatase (AP) labeled anti-fluorescein antibodies and horseradish peroxidase (POD) labeled anti-digoxigenin antibodies to determine whether either or both of the allele-specific probes have been covalently linked to the biotinylated probes during the ligation assay.

Figure 1. When typing a single nucleotide substitution, the allele-specific primers in the ligation reaction differ only by the nucleotide found at their 3'-end and the hapten group that marks their 5'-end. By labeling each of the allele-specific primers with a different hapten, i.e. fluorescein and digoxigenin, and labeling the common joining primer with a molecule that can be captured (biotin), diallelic sequence variations can be typed together in a single well of a microtiter plate and detected using ELISA analysis.

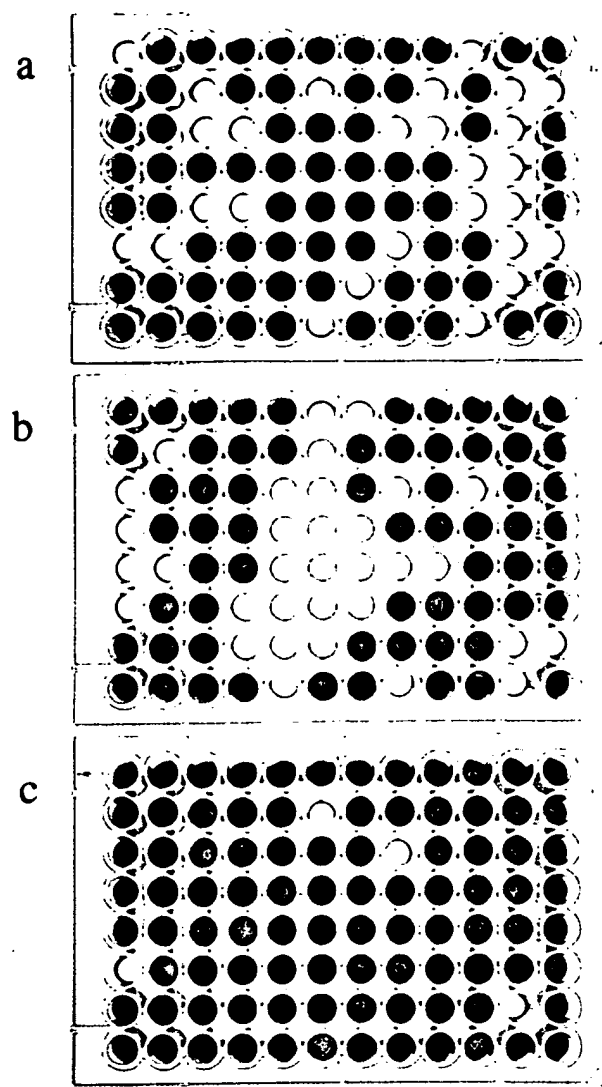


Figure 2. OLA genotyping of STS160 PCR products amplified from 96 samples. (a) Wells containing the fluorescein reporter (allele 1) produce a deep magenta color while negative reactions (no ligation with fluorescein-labeled primer) do not result in a colored product and remain clear. (b) The alkaline phosphate substrate is washed away, and the peroxidase substrate that detects the digoxigenin-labeled probe is added to the wells of the same plate. If digoxigenin is present (allele 2 in the product), the wells turn a brilliant blue color. In the absence of digoxigenin, the wells remain clear. (c) A photographic overlap of the results from (a) and (b). Homozygotes for fluorescein reporter (allele 1) appear red in the overlap, homozygotes for the digoxigenin reporter (allele 2) appear brilliant blue in the overlap, heterozygotes (the presence of both alleles, allele 1 and 2) appear purple while negative controls (water blanks in the PCR, negative for PCR and OLA) remain clear in the overlap.

In this approach, the ligation primers are hybridized to the PCR target in solution, and if there is perfect base-pair complementarity between the primers and PCR target, the two ligation primers (the hapten labeled primer(s) and the biotinylated joining primer) become covalently linked to one another by DNA ligase. If there is a mismatch between the primers and target, the primers are not joined during the assay. After ligation, the primers are captured on streptavidin-coated microtiter plates and an ELISA for the two allele-specific haptens is performed to determine which primer(s) remains attached to the plates after washing away the unligated

materials (Fig. 1). In the ELISA phase of the assay, specific anti-hapten antibodies to fluorescein and digoxigenin, each labeled with a different enzyme reporter (alkaline phosphatase (AP) and horseradish peroxidase (POD), respectively), are applied to detect the outcome of the ligation assay. Specific substrates for each of these reporter enzymes are sequentially applied to the plate to detect which haptens (fluorescein marking allele 1, or digoxigenin marking allele 2) are present in the wells (Fig. 1). Substrates cannot be combined because each enzyme has different pH and co-factor requirements for optimal color production.

We have genotyped several thousand DNA samples using multiplexed OLA. An example of our results is shown in Figure 2 using PCR products amplified from a polymorphic region of the human genome (STS160). As shown in Figure 2a, microtiter wells containing ligated products marked by the fluorescein hapten (allele 1) produce a deep magenta color when the alkaline phosphatase substrate is added to the microtiter plate. After reading in a microplate spectrophotometer and washing, a horseradish peroxidase substrate is added to the microtiter plate. This gives rise to a brilliant blue color if ligated products containing the digoxigenin-labeled primer (allele 2) are present in the wells (Fig. 2b). Although the color products can be observed visually, they are also read on a microplate reader. The genotype of the sample can then be automatically obtained by overlapping the numerical absorbance readings taken with the two substrates for each well using a computer program. To demonstrate this visually, slides of the color products formed by the alkaline phosphatase and horseradish peroxidase substrates from Figure 2a and b were overlapped and printed in Figure 2c. The four possible outcomes of this typing system are easily observed in this example: (i) wells producing only a red product indicating the PCR product was homozygous for allele 1, (ii) wells producing only a brilliant blue product indicating the PCR product was homozygous for allele 2, (iii) wells showing a deep purple color which is produced by the presence of both the red and blue products and indicates that the PCR product was heterozygous for allele 1 and 2, and (iv) no color production by either substrate which indicates the absence of a PCR product (in this example, four PCR water blanks placed among the DNA samples), or in the presence of a PCR product, a failed ligation assay. In checking negative ligation events, PCR failure has proven to be the most common cause of assay failure (~99%).

High signal-to-noise ratios (>10 to 1) are achieved with this system because two levels of specificity are applied in sample analysis. PCR followed by OLA. This greatly simplifies data interpretation since positive and negative assays are easily distinguished visually (Fig. 2) as well as numerically (Fig. 3). When absorbance readings for the two substrates are plotted, the four potential outcomes form distinct clusters (Fig. 3). In analyzing more than 8000 ELISA reactions (data not shown), we found that negative assays consistently yield optical absorbances ≤ 0.150 (range = 0.000 to 0.148, mean ± 1 SD = 0.003 ± 0.013). Therefore, we routinely apply this threshold in data analysis as shown in Table 1, and find it is the simplest and most accurate approach to calling a positive ($OD > 0.150$) or a negative ($OD \leq 0.150$) reaction.

By automating the process of allele calling, we have been able to focus greater attention on error analysis. We found that when two alleles are typed in separate microtiter wells (12), allele dropout (a heterozygous sample typing as a homozygous sample due to the failure of one of the two ligation reactions) is the

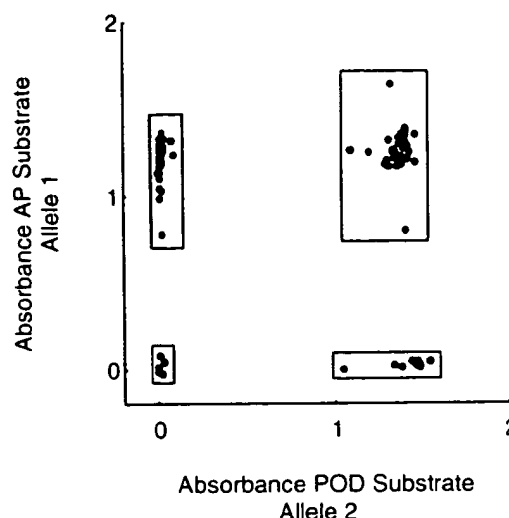


Figure 3. Absorbances obtained with microplate spectrophotometer following multiplex OLA analysis of 96 samples amplified from a polymorphic region in the human LPL gene. When absorbance readings are plotted, the four assay outcomes form distinct clusters (that have been boxed) based on genotype with genotypes of 0 at the origin, homozygotes for allele 1 close to the y-axis, homozygotes for allele 2 near to the x-axis and heterozygotes between the two axes.

greatest single contributor to typing error ($>90\%$). However, the dropout rate from locus to locus does vary (0.2% to ~1%, data not shown). Theoretically, typing two alleles in a single well should eliminate the majority of these errors since both ligation reactions in the well would fail and no genotype would be obtained. To test this hypothesis, we took a diallelic locus (SRD5A1) with one of the highest rates of allele dropout and typed the entire CEPH pedigree twice in separate wells. The rate of allele dropout when each allele was typed in a separate well was nine failed ligation reactions in 2032 wells. This yielded nine erroneous genotypes in 1016 (0.89%). When the same PCR products were retyped using the multiplexed approach, allele dropout occurred in only one well, or 1 erroneous genotype in 1016 (0.01%, a 9-fold decrease in dropout error due to the failure of one of the two allelic reactions). Similar results have been obtained with other markers (data not shown). Therefore, multiplex testing significantly improves the accuracy of genotyping by PCR/OLA.

The ability to type two alleles in a single microtiter well also increases the throughput of genotyping by PCR/OLA and doubles the number of genotypes that can be performed in a single day (>1000 genotypes/day/person with minimal automation, 12). Because a significant amount of automation is available for processing large numbers of microtiter plates/day, with the appropriate instrumentation, even higher throughputs are feasible (10 000 genotypes/day, 19). In addition to increasing throughput, this system also reduces genotyping costs since fewer reagents are utilized. We estimate that multiplex OLA with ELISA-based detection currently costs ~28 cents/genotype and combined with PCR, total costs are <50 cents/genotype. The one additional cost factor is the generation of the hapten-modified and phosphorylated primers, approximately \$250 for each primer set, but these costs are rapidly decreasing. Considering that more than a million assays can be performed from a single synthesis, the large-scale application of individual primer sets also significantly reduces the

primer cost per genotype (>1000 assays, <25 cents for primers/genotype; >10 000 assays, <2.5 cents/genotype).

Table 1. Absorbance readings and genotype assignment in multiplex OLA for variants in the SRD5A1 gene

AP Substrate Allele 1	POD Substrate Allele 2	Genotype ^a
0.011 ^b	1.684 ^c	22
0.018	1.916	22
1.343	0.005	11
1.309	0.021	11
0.796	1.317	12
0.843	1.286	12
1.067	1.422	12
1.395	0.957	12

^aGenotypes are assigned by applying a filter that scores positives (>0.150) and negatives (≤ 0.150) based on absorbance readings. When allele 1/allele 2 = +/-, a genotype of 11 is assigned while -/+, +/- and -/- are assigned genotypes of 22, 12 and 0, respectively.

^bAbsorbance at 495 nm.

^cAbsorbance at 655 nm.

There are several advantages to combined approaches to genotyping such as PCR/OLA. First, PCR is reliable and specific but can sometimes generate spurious products. Therefore, methods relying solely on fragment size may sometimes be difficult to interpret. Since OLA evaluates internal DNA sequences, its outcome is not influenced by the formation of secondary amplification products. This extra specificity is particularly important in large-scale applications of PCR since it allows the stringency of the PCR itself to be relaxed to the point where PCR failure becomes rare. Additionally, ELISA-based ligation assays are very sensitive since the readout uses another form of enzyme-based amplification. We have found that accurate genotypes can be obtained with <2 ng of a PCR product (data not shown).

Currently, we are testing whether additional haptens and reporter enzymes can be applied to achieve even higher levels of multiplexing in colorimetric formats while maintaining the high signal-to-noise ratios and sensitivity of the assay. However, even

in its present format, multiplex typing of diallelic DNA variations by PCR/OLA offers many advantages for high throughput genotyping in terms of its specificity, sensitivity and accuracy using a system that can be easily adapted to different applications requiring large-scale DNA typing (4,5,12).

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